SUMMARY OF THE INVENTION:

Calf-chymosin gene is isolated preferably from the fourth stomach of milk fed calf tissues. Recombinant calf-chymosin is produced by cloning chymosin gene with bacterial expression vector PET21b and is transformed to E-coli strain. This E-coli strain containing recombinant calf-chymosin gene is fermented under suitable conditions preferably in a culture medium developed by us. This medium contains the following

Peptone – 12g/l

Yeast Extract – 24g/l

Sodium chloride – 10g/l

Prochymosin produced during fermentation is subjected to denaturation by increasing the pH of the medium to 10-11. The suspension then diluted and the pH reduced to about 8 for effective renaturation of the protein. The prochymosin thus obtained is then acidified for activation and is further processed.

This invention relates to a process for producing recombinant calf-chymosin which comprises the steps of isolating calf-chymosin gene, cloning the same in bacterial expression vector PET 21b, transforming said cloned vector into cells of E-coli, fermenting said E-coli strains to produce pro-chymosin, converting said prochymosin to chymosin and subsequently recovering the recombinant calf chymosin.

[REPLACEMENT SHEET]

This invention also includes recombinant calf-chymosin having the following amino acid sequence:

```
   MetAlaSerIle ThrArgIle ProLeuTyr LysGlyLysSer LeuArgLys AlaLeuLys
 1 ATGGCTAGCA TCACTAGGAT CCCTCTGTAC AAAGGCAAGT CTCTGAGGAA GGCGCTGAAG
   TACCGATCGT AGTGATCCTA GGGAGACATG TTTCCGTTCA GAGACTCCTT CCGCGACTTC
   GluHisGlyLeu LeuGluAsp PheLeuGln LysGlnGlnTyr GlyIleSer SerLysTyr
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   CTCGTACCCG AAGACCTCCT GAAGGACGTC TTTGTCGTCA TACCGTAGTC GTCGTTCATG
   SerGlyPheGly GluValAla SerValPro LeuThrAsnTyr LeuAspSer GlnTyrPhe
121 TCCGGCTTCG GGGAGGTGGC CAGCGTGCCC CTGACCAACT ACCTGGATAG TCAGTACTTT
   AGGCCGAAGC CCCTCCACCG GTCGCACGGG GACTGGTTGA TGGACCTATC AGTCATGAAA
   GlyLysIleTyr LeuGlyThr ProProGln GluPheThrVal LeuPheAsp ThrGlySer
181 GGGAAGATCT ACCTCGGGAC CCCGCCCCAG GAGTTCACCG TGCTGTTTGA CACTGGCTCC
   CCCTTCTAGA TGGAGCCCTG GGGCGGGGTC CTCAAGTGGC ACGACAAACT GTGACCGAGG
   SerAspPheTrp ValProSer IleTyrCys LysSerAsnAla CysLysAsn HisGlnArg
241 TCTGACTTCT GGGTACCCTC TATCTACTGC AAGAGCAATG CCTGCAAAAA CCACCAGCGC
   AGACTGAAGA CCCATGGGAG ATAGATGACG TTCTCGTTAC GGACGTTTTT GGTGGTCGCG
   PheAspProArg LysSerSer ThrPheGln AsnLeuGlyLys ProLeuSer IleHisTyr
301 TTCGACCCGA GAAAGTCGTC CACCTTCCAG AACCTGGGCA AGCCCCTGTC TATCCACTAC
   AAGCTGGGCT CTTTCAGCAG GTGGAAGGTC TTGGACCCGT TCGGGGACAG ATAGGTGATG
   GlyThrGlyLys MetGlnGly IleLeuGly TyrAspThrVal ThrValSer AsnIleVal
361 GGGACAGGCA AGATGCAGGG GATCCTGGGC TATGACACCG TCACTGTCTC CAACATTGTG
   CCCTGTCCGT TCTACGTCCC CTAGGACCCG ATACTGTGGC AGTGACAGAG GTTGTAACAC
   AspIleGlnGln ThrValVal LeuSerThr GlnGluProGly AspValPhe ThrTyrAla
421 GACATCCAGC AGACAGTAGT CCTGAGCACC CAGGAGCCCG GGGACGTCTT CACCTATGCC
   CTGTAGGTCG TCTGTCATCA GGACTCGTGG GTCCTCGGGC CCCTGCAGAA GTGGATACGG
   GluPheAspGly IleLeuGly MetAlaTyr ProSerLeuAla SerGluVal LeuAspThr
481 GAATTCGACG GGATCCTGGG GATGGCGTAC CCCTCGCTGG CCTCAGAAGT ACTCGATACC
   CTTAAGCTGC CCTAGGACCC CTACCGCATG GGGAGCGACC GGAGTCTTCA TGAGCTATGG
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   CCGAAACTGT TGTACTACTT GTCCGTGGAC CACCGGGTTC TGCACAAGAG CCAAATGTAC
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   CTGTCCTTAC CCGTCCCTTT GTACAAATGG GACCCCCGGT AGCTGGGCAG GATGATGTGT
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   CCCAGGGACG TGACCCACGG GCACTGTCAC GTCGTCATGA CCGTCAAGTG ACACCTGTCA
   ValThrIleSer GlyValVal ValAlaCys GluGlyGlyCys GlnAlaIle LeuAspThr
721 GTCACCATCA GCGGTGTGGT TGTGGCCTGT GAGGGTGGCT GTCAGGCCAT CCTGGACACG
   CAGTGGTAGT CGCCACACCA ACACCGGACA CTCCCACCGA CAGTCCGGTA GGACCTGTGC
   GlyThrSerLys LeuValGly ProSerSer AspIleLeuAsn IleGlnGln AlaIleGly
781 GGCACCTCCA AGCTGGTCGG GCCCAGCAGC GACATCCTCA ACATCCAGCA GGCCATTGGA
   CCGTGGAGGT TCGACCAGCC CGGGTCGTCG CTGTAGGAGT TGTAGGTCGT CCGGTAACCT
   AlaThrGlnAsn GlnTyrAsp GluPheAsp IleAspCysAsp AsnLeuSer TyrMetPro
841 GCCACACAGA ACCAGTACGA TGAGTTTGAC ATCGACTGCG ACAACCTGAG CTACATGCCC
   CGGTGTGTCT TGGTCATGCT ACTCAAACTG TAGCTGACGC TGTTGGACTC GATGTACGGG
   ThrValValPhe GluIleAsn GlyLysMet TyrProLeuThr ProSerAla TyrThrSer
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   GlnAspGlnGly PheCysThr SerGlyPhe GlnSerGluAsn HisSerGln LysTrpIle
```


[REPLACEMENT SHEET]

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     LeuGlyAspVal PheIleArg GluTyrTyr SerValPheAsp ArgAlaAsn AsnLeuVal
1021 CTGGGGGATG TTTTCATCCG AGAGTATTAC AGCGTCTTTG ACAGGGCCAA CAACCTCGTG
     GACCCCCTAC AAAAGTAGGC TCTCATAATG TCGCAGAAAC TGTCCCGGTT GTTGGAGCAC
     GlyLeuAlaLys AlaIle***
1081 GGGCTGGCCA AAGCCATCTG A
     CCCGACCGGT TTCGGTAGAC T
```

In the above sequence, amino acids shown in red indicate sequence variation of chymosin gene of our invention compared to the reported and published sequence.

[REPLACEMENT SHEET]

PCR amplification of prepro chymosin was performed using the 50ng of 1st strand cDNA with a reverse primer (5'-TGT GGG GAC AGT GAG GTT CTT GGT C-3'), and a forward primer (5'-ATG AGG TGT CTC GTG GTG CTA CTT-3') in a thermal cycler programmed as (step 1: 95-5'; step 2: 94-30sec.; step 3: 54-30sec; step 4: 72-1min; step 5: go to step 2 34 times; step 6: 72-7min; step 7: end). The PCT reaction when analyzed on 1.0% agarose gel showed an amplified band of 1.2kb. The 1.2kb fragment was cut with a sterile blade and the gel slice was dissolved in 500µl of Tris saturated phenol was added and left in liquid nitrogen for a few min. The microcentrifuge tube was allowed to come to room temperature and centrifuged for 5min at 12,000rpm, 4°C. The upper aqueous phase was extracted with phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and DNA was precipitated with 1/10th volume sodium acetate and 2.5 volume ethanol at –70°C for 1 h. DNA was precipitated at 15,000rpm for 15 min. The pellet was dried and dissolved in sterile distilled water. This eluted 1.2kb fragment was ligated at SmaI site of pBSSK+ plasmid, which was then transformed in to TOP10 cells of E.coli. The recombinant clones were selected (blue white screening) and checked with restriction digestion analysis of the plasmids. Recombinant plasmid was taken as a template and a PCR was performed using a forward primer (5'-GAT ATA CAT ATG GCT AGC ATC ACT AGG ATC CCT CTG TAC-3') and reverse primer (5'-GCA GTA AGC TTG ACA GTG AGG TTC CTT GGT CAG CG-3') containing Nde 1 and Hind III sites. An amplified band of 1098bp was observed when the PCR product was analyzed on 1.0% agarose gel. This amplified fragment of 1098bp was eluted from the gel and ligated in pET21b expression vector at Nde 1 and Hind III sites and transformed in to BL21 cells of E.coli for the expression of the chymosin gene.

[REPLACEMENT SHEET]

CLAIMS:

1. A process for producing recombinant calf-chymosin which comprises the steps of isolating calf-chymosin gene, cloning the same in bacterial expression vector PET21b, transforming said cloned vector into cells of E.coli, fermenting said E.coli strains to produce pro-chymosin, converting said pro-chymosin to chymosin and subsequently recovering the recombinant calf-chymosin.

2. The process as claimed in claim 1, wherein calf-chymosin gene is obtained by isolating RNA from the fourth stomach of calf tissue, synthesising a first strand of cDNA therefrom by treating the same with a reverse primer such as 5'-TGT GGG GAG AGT GAG GTT CTT GGT C-3' and then with a forward primer such as 5'-ATG AGG TGT CTC GTG GTG CTA CTT 3 and with a reverse primer such as 5'TGT GGT GAC AGT GAG GTT CTT GGT C-3'.

3. The process as claimed in claims 1 and 2 wherein said C DNA is ligated at small site of pBSSK+ plasmid and then transformed into TOP 10 cells of E-coli.

4. The process as claimed in claim 3 wherein said recombinant clones were identified and treated with a forward primer such as 5'-GAT ATA CAT ATG GCT AGC ATC ACT AGG ATC CCT CTG TAC 3' and reverse primer such as 5' GCA GTA AGC TTG ACA GTG TTC CTT GGT CAG CG-3' containing Nde I and Hind III sites to obtain an amplified fragment.

5. The process as claimed in claim 4 wherein said amplified fragment is transformed into cells of E.coli for expressing said chymosin gene.

{REPLACEMENT SHEET}

6. The process as claimed in any of the preceding claims wherein said E.coli cells containing recombinant calf chymosin gene is fermented in a medium containing 12g/L peptone, 24g/L of yeast extract and 10g/L of sodium chloride in the presence of supplements for fermentation and the suspended cells produced on completion of fermentation is lysed, chilled and pH adjusted to 8 before incubating at room temperature and the supernatent containing prochymosin is separated.

7. The process as claimed in claim 6, wherein the pH of said prochymosin containing supernatent is adjusted to 2 at room temperature and further incubated for about 6 hrs with gentle stirring and filtered.

8. The process as claimed in claim 7 wherein the pH of said filtrate is adjusted to about 5 and further incubated, filtered and treated with a solution containing sodium benzoate and thereafter a solution containing and sodium chloride to activate prochymosin to chymosin.

9. The process as claimed in claim 8 wherein the filtrate obtained after the addition of sodium benzoate solution is treated with a solution of sodium chloride under stirring and cooking, and the precipitate suspended in a chilled solution of 0.2M glycine with 0.001M EDTA and thereafter treated with 0.23% solution of sodium benzoate and stored under cooling.

10. The process as claimed in claim 9 wherein said chymosin obtained is formulated with 10% of sodium chloride and 0.2% of Trehalose.

REPLACEMENT SHEET

11. Recombinant calf-chymosin having the following amino acid sequence:

```
      MetAlaSerIle ThrArgIle ProLeuTyr LysGlyLysSer LeuArgLys AlaLeuLys
    1 ATGGCTAGCA TCACTAGGAT CCCTCTGTAC AAAGGCAAGT CTCTGAGGAA GGCGCTGAAG
      TACCGATCGT AGTGATCCTA GGGAGACATG TTTCCGTTCA GAGACTCCTT CCGCGACTTC
      GluHisGlyLeu LeuGluAsp PheLeuGln LysGlnGlnTyr GlyIleSer SerLysTyr
   61 GAGCATGGGC TTCTGGAGGA CTTCCTGCAG AAACAGCAGT ATGGCATCAG CAGCAAGTAC
      CTCGTACCCG AAGACCTCCT GAAGGACGTC TTTGTCGTCA TACCGTAGTC GTCGTTCATG
      SerGlyPheGly GluValAla SerValPro LeuThrAsnTyr LeuAspSer GlnTyrPhe
  121 TCCGGCTTCG GGGAGGTGGC CAGCGTGCCC CTGACCAACT ACCTGGATAG TCAGTACTTT
      AGGCCGAAGC CCCTCCACCG GTCGCACGGG GACTGGTTGA TGGACCTATC AGTCATGAAA
      GlyLysIleTyr LeuGlyThr ProProGln GluPheThrVal LeuPheAsp ThrGlySer
  181 GGGAAGATCT ACCTCGGGAC CCCGCCCCAG GAGTTCACCG TGCTGTTTGA CACTGGCTCC
      CCCTTCTAGA TGGAGCCCTG GGGCGGGGTC CTCAAGTGGC ACGACAAACT GTGACCGAGG
      SerAspPheTrp ValProSer IleTyrCys LysSerAsnAla CysLysAsn HisGlnArg
  241 TCTGACTTCT GGGTACCCTC TATCTACTGC AAGAGCAATG CCTGCAAAAA CCACCAGCGC
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      AAGCTGGGCT CTTTCAGCAG GTGGAAGGTC TTGGACCCGT TCGGGGACAG ATAGGTGATG
      GlyThrGlyLys MetGlnGly IleLeuGly TyrAspThrVal ThrValSer AsnIleVal
  361 GGGACAGGCA AGATGCAGGG GATCCTGGGC TATGACACCG TCACTGTCTC CAACATTGTG
      CCCTGTCCGT TCTACGTCCC CTAGGACCCG ATACTGTGGC AGTGACAGAG GTTGTAACAC
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      ValThrIleSer GlyValVal ValAlaCys GluGlyGlyCys GlnAlaIle LeuAspThr
  721 GTCACCATCA GCGGTGTGGT TGTGGCCTGT GAGGGTGGCT GTCAGGCCAT CCTGGACACG
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      GlyThrSerLys LeuValGly ProSerSer AspIleLeuAsn IleGlnGln AlaIleGly
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      CGGTGTGTCT TGGTCATGCT ACTCAAACTG TAGCTGACGC TGTTGGACTC GATGTACGGG
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[REPLACEMENT SHEET]

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1021 CTGGGGGATG TTTTCATCCG AGAGTATTAC AGCGTCTTTG ACAGGGCCAA CAACCTCGTG
     GACCCCCTAC AAAAGTAGGC TCTCATAATG TCGCAGAAAC TGTCCCGGTT GTTGGAGCAC
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1081 GGGCTGGCCA AAGCCATCTG A
     CCCGACCGGT TTCGGTAGAC T
```

13. Recombinant calf-chymosin when produced by a process according to any of the preceding claims.

{REPLACEMENT SHEET}

ABSTRACT

Calf-chymosin containing 1098 p coding for 366 amino acids was isolated and cloned into a bacterial expression vector pET 21b. This strain is fermented and the protein is precipitated by addition of alkali. Active form is recovered by renaturation and purified under low pH.

[R&PLACEMENT SHEET]

SEQUENCE LISTING

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Sudershan Biotech Limited –

<120> Recombinant Calf Chymosin and a process for producing the same

<130> PCT0602

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            20                  25                  30

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        35                  40                  45

Val Pro Leu Thr Asn Tyr Leu Asp Ser Gln Tyr Phe Gly Lys Ile Tyr
    50                  55                  60

Leu Gly Thr Pro Pro Gln Glu Phe Thr Val Leu Phe Asp Thr Gly Ser
65                  70                  75                  80

Ser Asp Phe Trp Val Pro Ser Ile Tyr Cys Lys Ser Asn Ala Cys Lys
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Asn His Gln Arg Phe Asp Pro Arg Lys Ser Ser Thr Phe Gln Asn Leu
            100                 105                 110

Gly Lys Pro Leu Ser Ile His Tyr Gly Thr Gly Lys Met Gln Gly Ile
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Leu Gly Tyr Asp Thr Val Thr Val Ser Asn Ile Val Asp Ile Gln Gln
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[REPLACEMENT SHEET]

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        195                 200                 205

Phe Thr Leu Gly Ala Ile Asp Pro Ser Tyr Tyr Thr Gly Ser Leu His
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Trp Val Pro Val Thr Val Gln Gln Tyr Trp Gln Phe Thr Val Asp Ser
225                 230                 235                 240

Val Thr Ile Ser Gly Val Val Val Ala Cys Glu Gly Gly Cys Gln Ala
                245                 250                 255

Ile Leu Asp Thr Gly Thr Ser Lys Leu Val Gly Pro Ser Ser Asp Ile
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Leu Asn Ile Gln Gln Ala Ile Gly Ala Thr Gln Asn Gln Tyr Asp Glu
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Phe Asp Ile Asp Cys Asp Asn Leu Ser Tyr Met Pro Thr Val Val Phe
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Glu Ile Asn Gly Lys Met Tyr Pro Leu Thr Pro Ser Ala Tyr Thr Ser
305                 310                 315                 320

Gln Asp Gln Gly Phe Cys Thr Ser Gly Phe Gln Ser Glu Asn His Ser
                325                 330                 335

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caggaccagg gcttctgtac cagtggcttc cagagtgaaa atcattccca gaaatggatc 1020

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[REPLACEMENT SHEET]

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[REPLACEMENT SHEET]